

AD-A259 579

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3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE

Fluoxetine Hydrochloride Enhances In Vitro Susceptibility to Chloroquine in Resistant *Plasmodium falciparum*

6. AUTHOR(S)

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5. FUNDING NUMBERS

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Walter Reed Army Institute of Research
Washington, DC 20307-51008. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research & Development Command
Ft. Detrick, Frederick, MD 21702-501210. SPONSORING/MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

DTIC

SELECTED
JAN 12 1993

12a. DISTRIBUTION/AVAILABILITY STATEMENT

APPROVED FOR PUBLIC RELEASE
DISTRIBUTION UNLIMITED

12b. DISTRIBUTION CODE

S B D

13. ABSTRACT (Maximum 200 words)

The emergence of chloroquine resistance in *Plasmodium falciparum* has necessitated the development of alternate strategies for chemotherapy and chemoprophylaxis. One approach has been the identification of drugs that do not possess any intrinsic antimalarial activity when used alone but that potentiate the effect of currently available antimalarial drugs, such as chloroquine. We identified fluoxetine hydrochloride (Prozac), a commonly prescribed antidepressant, as another resistance modulator for drug-resistant *P. falciparum*. Studies with chloroquine-resistant clones and isolates from various geographical locations confirmed our initial observations with a chloroquine-resistant *P. falciparum* clone, W2. Fluoxetine concentrations of 500 nM were found to effectively modulate chloroquine resistance by 66% in clone W2. In comparison, verapamil at similar concentrations was observed to modulate chloroquine resistance in clone W2 by 61%. Neither fluoxetine nor verapamil was observed to possess any innate antimalarial activity. These data augment the current description of the chloroquine resistance phenotype and may provide additional insights into lead-directed synthesis of new antimalarial drugs.

14. SUBJECT TERMS

Fluoxetine hydrochloride; chloroquine resistance;
Plasmodium falciparum

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT18. SECURITY CLASSIFICATION
OF THIS PAGE19. SECURITY CLASSIFICATION
OF ABSTRACT

20. LIMITATION OF ABSTRACT

Fluoxetine Hydrochloride Enhances In Vitro Susceptibility to Chloroquine in Resistant *Plasmodium falciparum*

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Received 24 August 1992/Accepted 28 September 1992

The emergence of chloroquine resistance in *Plasmodium falciparum* has necessitated the development of alternate strategies for chemotherapy and chemoprophylaxis. One approach has been the identification of drugs that do not possess any intrinsic antimalarial activity when used alone but that potentiate the effect of currently available antimalarial drugs, such as chloroquine. We identified fluoxetine hydrochloride (Prozac), a commonly prescribed antidepressant, as another resistance modulator for drug-resistant *P. falciparum*. Studies with chloroquine-resistant clones and isolates from various geographical locations confirmed our initial observations with a chloroquine-resistant *P. falciparum* clone, W2. Fluoxetine concentrations of 500 nM were found to effectively modulate chloroquine resistance by 66% in clone W2. In comparison, verapamil at similar concentrations was observed to modulate chloroquine resistance in clone W2 by 61%. Neither fluoxetine nor verapamil was observed to possess any innate antimalarial activity. These data augment the current description of the chloroquine resistance phenotype and may provide additional insights into lead-directed synthesis of new antimalarial drugs.

Malaria is a significant source of morbidity in the world, with an estimated annual prevalence of 270 million infections (19). It is a serious impediment to economic and agricultural growth and development, particularly in less developed countries. At present, the quinoline ring-containing drugs remain the most efficacious drugs for chemoprophylaxis and chemotherapy of *Plasmodium falciparum* malaria. Chloroquine, a 4-aminoquinoline, has been the drug of choice for several decades. However, the increasing prevalence and the degree of chloroquine resistance in regions endemic for *P. falciparum* malaria have substantially compromised its clinical utility. Although more than 350,000 compounds have been evaluated for their antimalarial activities at the Walter Reed Army Institute of Research, few new leads for drug development have been identified. Despite the development of mefloquine, halofantrine, and artemisinin as new blood schizonticides, few effective drugs with enhanced antimalarial activity, reduced cost, and low toxicity exist. In order to preserve the efficacies of current and future chemotherapeutic agents, alternative drug strategies must continually be developed, evaluated, and implemented. One approach that merits further investigation and possible implementation has been the identification of drugs that, at subinhibitory concentrations, modulate chloroquine resistance. Such drugs include verapamil (14), desipramine (3), chlorpromazine (12), ketotifen (1), tetrandrine (20, 21), and cyproheptadine (16).

Our rationale for screening fluoxetine hydrochloride (Prozac) as a potential modulator was based on our previous observations that various compounds with neuroleptic activ-

ity, desipramine (3) and chlorpromazine (12), are effective modulators of chloroquine resistance; in contrast, penfluridol (13, 17) has been found to potentiate mefloquine, halofantrine, and artemisinin. In addition, fluoxetine met certain structural criteria previously established for resistance modulators. Lipid solubility, two planar aromatic rings, a cationic charge, and a tertiary nitrogen were found to be important characteristics for reversal modulators (2, 22). Whereas fluoxetine fulfilled these criteria, it is worth noting that the structure is unlike those of other reported modulators of chloroquine resistance, the tricyclic antidepressants (desipramine), the phenothiazines (chlorpromazine), or the calcium channel antagonists (verapamil) (Fig. 1). We present in vitro data that identify fluoxetine as a reversal modulator for chloroquine-resistant *P. falciparum* malaria.

MATERIALS AND METHODS

Parasite clones and isolates. Several well-characterized clones and isolates of *P. falciparum* with different susceptibility profiles were used for the drug assays described here. The clones W2 (15) and D6 (15) are reference clones from Indochina III and Sierra Leone isolates, respectively. The threshold values for chloroquine susceptibility and resistance were determined by using the reference chloroquine-susceptible clone D6 (50% inhibitory concentration [IC₅₀], ≤3 ng/ml). IC₅₀s greater than 4 ng of chloroquine per ml were considered resistant. Clone GA3 is a clone of the GH2 isolate obtained from Thailand. Clone 306 is a clone of the IEC 51/84 isolate from Brazil. Nigeria 60 is a patient isolate recently collected from Africa. Clones GA3, 306, and W2 and isolate Nigeria 60 are chloroquine resistant.

Drug assays. The susceptibilities of each clone to the tested drugs were evaluated by using a modification of the semiautomated microdilution technique (7). Chloroquine diphosphate, fluoxetine hydrochloride, and verapamil hy-

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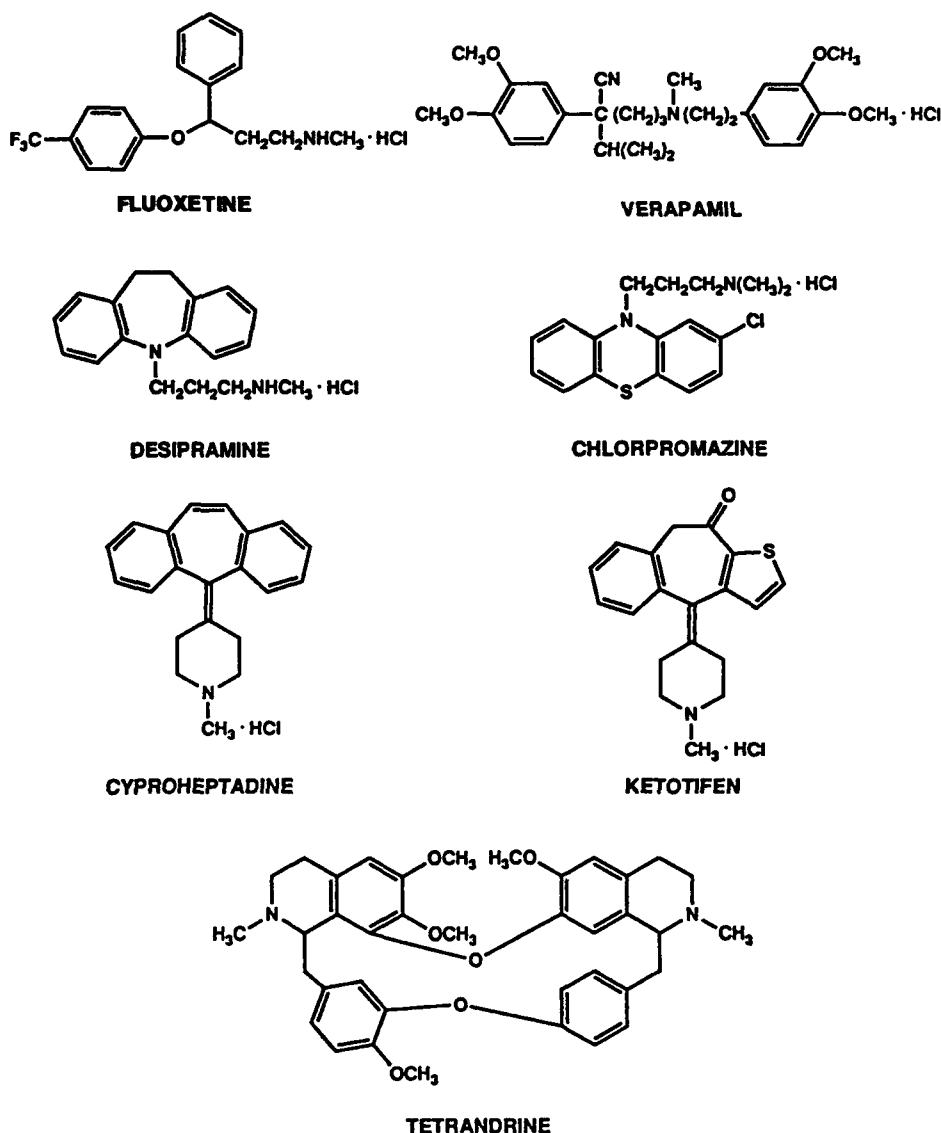


FIG. 1. Structures of fluoxetine and other drugs that have been reported to modulate chloroquine resistance in *P. falciparum*. Fluoxetine is not structurally similar to other previously reported reversal modulators.

drochloride were obtained from an inventory of drugs maintained by the Walter Reed Army Institute of Research. Drug assays were performed in 96-well microtiter plates with 1% erythrocyte suspensions at 0.2 to 0.5% parasitemia in RPMI 1640 supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 32 mM NaHCO₃, and 10% human plasma. Microtiter plates were prepared with serial dilutions of drug to determine IC₅₀s. Each plate containing parasites and drug was incubated at 37°C in an airtight Plexiglas box in the presence of 5% oxygen–5% carbon dioxide–90% nitrogen. After 24 h of incubation, cultures were labeled with [³H]hypoxanthine (NEN Dupont) and were then incubated for an additional 18 to 20 h prior to harvesting. Incorporation of radiolabel in each well was determined by scintillation spectrophotometry, and computer-generated concentration-response curves were analyzed by nonlinear regression. The susceptibilities of the parasites to the antimalarial drugs alone and in combination with

subinhibitory concentrations of the reversal agents were compared to ascertain the degree of modulation. In order to evaluate fluoxetine's modulation of chloroquine resistance, simultaneous studies were performed with verapamil, a drug whose reversal activity in *P. falciparum* has been well documented (14). The IC₅₀s of each drug alone and of various drug combinations were determined.

Modulation of the susceptibility of the parasite to chloroquine singularly and in the presence of the resistance modulator at predetermined concentrations was expressed as the response modification index (RMI) (12). RMIs were calculated by the following formula: $RMI = IC_{50(A,B)} / IC_{50(A)}$, where IC_{50(A)} is the chloroquine IC₅₀, and IC_{50(B)} represents the resistance modulator IC₅₀. The resistance modulator (B), by definition, possesses little, if any, intrinsic antimalarial activity. An RMI equivalent to 1.0 represents no change in the IC₅₀ upon addition of the resistance modulator at predetermined levels. An RMI of greater than 1.0 demonstrates an

TABLE 1. Reversal of chloroquine resistance in *P. falciparum* by verapamil

| Drug | IC ₅₀ (ng/ml [RMI]) for ^a | | | | |
|--------------------------------|---|--------------|---------------|---------------|----------------|
| | Clone D6 (S) | Clone W2 (R) | Clone GA3 (R) | Clone 306 (R) | Nigeria 60 (R) |
| Verapamil | 5,360 | 1,606.07 | 2,730 | >492 | 1,245 |
| Chloroquine | 2.89 (1.0) | 71.1 (1.0) | 22.09 (1.0) | 68.0 (1.0) | 66.3 (1.0) |
| Chloroquine with verapamil at: | | | | | |
| 62.5 nM | 2.69 (0.93) | 67.6 (0.95) | 20.2 (0.91) | 68.9 (1.01) | 59.0 (0.89) |
| 125 nM | 2.92 (1.01) | 51.1 (0.72) | 32.7 (1.48) | 70.0 (1.02) | 47.7 (0.72) |
| 250 nM | 2.79 (0.96) | 39.3 (0.55) | 13.0 (0.59) | 65.9 (0.97) | 20.0 (0.30) |
| 500 nM | 2.62 (0.91) | 27.5 (0.39) | 11.5 (0.52) | 60.7 (0.89) | 14.9 (0.22) |
| 1,000 nM | 2.73 (0.95) | 14.6 (0.21) | 6.0 (0.27) | 42.2 (0.62) | 6.84 (0.10) |

^a R, chloroquine resistant; S, chloroquine susceptible.

antagonistic effect between the resistance modulator and chloroquine, whereas an RMI of less than 1.0 represents potentiation or synergism.

RESULTS

The IC₅₀ responses of geographically diverse clones and isolates of *P. falciparum* to chloroquine alone and in combination with resistance modulators are presented in Tables 1 and 2. The response of the chloroquine-susceptible parasite clone D6 to the fluoxetine and chloroquine combination was found to be similar to that of the clone to verapamil and chloroquine (Tables 1 and 2, respectively). For either combination of drug and resistance modulator, there was no potentiation of chloroquine in the susceptible parasite. Indeed, the RMIs suggested that the effects of these modulators were additive for chloroquine (Tables 1 and 2). Nor did the reversal modulators used singly possess any significant antimalarial activity. These data for fluoxetine and verapamil are consistent with similar observations for other chloroquine reversal agents (3, 12-14).

In contrast, the data obtained for clone W2, the chloroquine-resistant parasite, suggested that fluoxetine is as effective as verapamil at reversing chloroquine resistance (Table 2). At concentrations from 62.5 to 1,000 nM, the RMIs for fluoxetine and verapamil in combination with chloroquine were not found to be different (1.06 to 0.18 and 0.95 to 0.21 at 1,000 nM, respectively) (Tables 1 and 2).

To confirm these observations, similar studies were performed with more recent clones and isolates from different geographical locations. It has been reported that responses to various reversal modulators are contingent upon the

antimalarial drug (chloroquine, mefloquine, etc.) and the origin of the parasite isolate (13). Therefore, clones and isolates obtained from Southeast Asia, Africa, and South America were evaluated. The degree of reversal as determined by the RMIs were similar in parasites of diverse geographical origin, with the exception of the South American clone, clone 306 (Tables 1 and 2).

DISCUSSION

Preservation of the clinical utility of chloroquine represents a significant concern and challenge for public health officials and clinicians. The rapid spread of drug resistance has resulted in a concerted effort to disseminate new drugs such as mefloquine, artemisinin, and halofantrine as alternatives for chemoprophylaxis and chemotherapy of *P. falciparum* malaria. However, with the notable exception of mefloquine, these drugs have not been licensed in all countries. Chloroquine remains the most attractive drug because of its availability and low cost. Therefore, we have been interested in identifying and developing drug and antimalarial drug combinations that may act to restore chloroquine activity in chloroquine-resistant parasites.

The mechanism of action of fluoxetine is not well characterized; however, it is presumed to be a serotonin uptake inhibitor (10). It is this aspect of the drug's proposed action that may have relevance to its effect on reversing chloroquine resistance. It has been demonstrated that in drug-resistant human cell lines the extrusion of cytotoxic compounds is mediated by an energy-dependent transport mechanism involving the P-170 glycoprotein encoded by *mdr1* (9). The *P. falciparum* equivalent of the human *mdr1*

TABLE 2. Reversal of chloroquine resistance in *P. falciparum* by fluoxetine

| Drug | IC ₅₀ (ng/ml [RMI]) for ^a : | | | | |
|---------------------------------|---|--------------|---------------|---------------|----------------|
| | Clone D6 (S) | Clone W2 (R) | Clone GA3 (R) | Clone 306 (R) | Nigeria 60 (R) |
| Fluoxetine | 2,000 | 1,500 | 1,359 | >346 | >346 |
| Chloroquine | 2.58 (1.0) | 86 (1.0) | 55.6 (1.0) | 36.5 (1.0) | 56.5 (1.0) |
| Chloroquine with fluoxetine at: | | | | | |
| 62.5 nM | 2.63 (1.02) | 91.2 (1.06) | 38.0 (0.68) | 20.1 (0.55) | 52.4 (0.93) |
| 125 nM | 2.62 (1.02) | 78.7 (0.92) | 21.1 (0.38) | 22.6 (0.62) | 35.7 (0.63) |
| 250 nM | 2.84 (1.10) | 45.0 (0.53) | 20.0 (0.36) | 18.0 (0.49) | 16.7 (0.30) |
| 500 nM | 3.82 (1.48) | 29.0 (0.34) | 18.0 (0.32) | 18.0 (0.49) | 12.9 (0.23) |
| 1,000 nM | 2.59 (1.0) | 15.8 (0.18) | 10.8 (0.19) | 7.2 (0.19) | 4.9 (0.09) |

^a R, chloroquine resistant; S, chloroquine susceptible.

gene, the *pfmdr1* gene, has been identified (8, 18) and localized to the parasitic digestive vacuole (6). However, no differences in the quantitation of the p-glycoprotein in chloroquine-susceptible and -resistant clones were observed. It has been reported that the accumulation of chloroquine is less in resistant parasites than it is in susceptible parasites because of an active efflux mechanism (11).

The proposed mechanism of reversal of resistance for fluoxetine and other modulators of *P. falciparum* drug resistance is unknown. Hypothetically, resistance modulators inhibit the extrusion of these cytotoxic compounds from the parasite by competition for a binding site on the P-170 glycoprotein. Presumably, this has been proposed as the mechanism of reversal in the human equivalent P-170 glycoprotein (4, 5). It is also possible that fluoxetine and other modulators perturb the integrity of the membrane or disrupt the kinetics of chloroquine uptake. The net effect is to increase the resident time and the accumulation of chloroquine in the parasite to cytotoxic levels.

It also was interesting that fluoxetine was effective at reversing chloroquine resistance in clones and isolates from different geographical origins and that the degree of reversal by verapamil was less than that achieved by fluoxetine in the South American parasite, clone 306 (Tables 1 and 2). We have previously observed (11a) that the extent of reversal of chloroquine resistance by verapamil and other modulators in South American clones and isolates was always less than that found in Southeast Asian resistant parasites. This finding may be due in part to the independent evolution of chloroquine resistance in South America and Southeast Asia. Genetic differences, namely, polymorphisms within *pfmdr1*, have previously been documented between South American and Southeast Asian parasites (8). These data warrant additional investigations with other South American clones and isolates. On the basis of these reversal modulator data, we postulate that the chloroquine resistance phenotype is similar throughout the world, although there may be subtle differences between geographical areas (Tables 1 and 2). This suggests that the genes responsible for mediating chloroquine resistance are similar, despite vast geographical distances, variant drug pressures, drug dissemination, or the independent evolution of chloroquine resistance in South America and Southeast Asia.

The levels of fluoxetine required to reverse chloroquine resistance may be within the chemotherapeutically achievable limits. Patients who were treated with 20 mg/day for 3 weeks, 40 mg/day during week 4, and 20 to 60 mg/day for weeks 5 and 6 had measurable fluoxetine levels of 60 to 453 ng/ml (173 to 1,310 nM) in serum (10). In resistant isolates and clones, the reversal of chloroquine resistance at 500 nM concentrations of fluoxetine ranged from 51 to 77% (Table 2). However, these *in vivo* data should not be taken as a recommendation for the immediate therapeutic use of fluoxetine in combination with chloroquine. The absence of *in vitro* data with this drug combination, the potential induction of neuropsychotic episodes, and the possible toxicity from increased deposition of chloroquine into tissues preclude this drug combination from being implemented clinically. However, additional evaluation for restricted utility in specific instances of severe chloroquine-resistant *P. falciparum* malaria warrants *in vivo* studies in murine and primate models. Similar *in vitro* and *in vivo* studies with desipramine, a tricyclic psychotropic drug, were found to be effective in reducing parasitemia (3). *In vivo* studies with fluoxetine in *Aotus* monkeys are in progress. In addition, structure-activity studies with analogs of fluoxetine may

identify potent resistance modulators without significant neuroleptic effects.

The continued identification of additional resistance modulators such as fluoxetine will assist in the efforts to exquisitely define the structure-activity relationships of these drugs and the drug resistance phenotype and may provide evidence for identifying the gene(s) and mechanisms involved in the resistance phenotype. The selection criteria for modulating antimalarial resistance may further be delineated on the basis of the general reversal activity of the drug, the geographical origin of the resistant parasite, and the specific resistance phenotype addressed.

ACKNOWLEDGMENTS

We thank Max Groggl, Malcolm Gardner, and Edwin O. Nuzum for critical review of the manuscript. We also thank Erich K. Lehnert for assistance in preparing the manuscript. We are grateful for the technical assistance of Renee L. Laukka. A. M. J. Oduola gratefully acknowledges the financial support of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the Rockefeller Foundation Biotechnology Career Fellowship.

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